

Analysis of intraspecific genetic variation, antioxidant and antibacterial activities of *Zingiber zerumbet*

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Abstract

Genetic variation of *Zingiber zerumbet* was determined by a RAPD technique using 7 arbitrary primers, and ITS, trnL intron and trnL-F spacer sequences. The RAPD results showed intraspecific genetic variation (mean $He \pm SD$: 0.2297 ± 0.0365 and % polymorphic bands: 62.86%). In addition, results of ITS, trnL intron and trnL-F spacer sequences revealed 856 variable sites, 451 nucleotide transitions and 147 nucleotide transversions. The intraspecific genetic variation was also detected in moderate level with the value of 1.032. Moreover, a neighbor-joining tree generated from RAPD was divided into two main groups, similar to the result of the gene sequence analysis. Significant differences in biological activities among tested samples of *Z. zerumbet* in the area were found. The antioxidant activities of the ethanolic extracts were determined by FIC and ABTS assay. It showed significantly different abilities in chelating ferrous ion and antioxidant activities (P -value < 0.05). Moreover, the ethanolic extracts of the tested samples were significantly different effective against both gram negative and positive bacteria (P -value < 0.05): *Escherichia coli*, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*.

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Keywords

Intraspecific genetic variation

Antioxidant activity

Antibacterial activity

Zingiber zerumbet

Introduction

Several Thai herbs have been reported for the benefit in medication, such as antioxidant and antibacterial properties (Thummajitsakul *et al.*, 2014). Among these, the herbaceous plants in family Zingiberaceae, genus *Zingiber* are traditional popular known in abundant medicinal values such as inflammation, diarrhea and indigestion (Wutthithamavet 1997; Bhuiyan *et al.*, 2009; Zakaria *et al.* 2010). Of these, *Zingiber zerumbet* is one of major members in the family Zingiberaceae well known as Haeo dam, Haeo Dang, Ka thue Pa or Ka thue. This species is widespread in hilly and tropical regions such as Cambodia, India, Thailand, Vietnam, China and Taiwan (Lim, 2014). *Z. zerumbet* was used as edible native ginger with tall of stem approximately 1-2 m, which there are rhizome that is part of stem in underground, leaves (approximately 25–35 cm long) and inflorescences (approximately 6–12 cm long) growing from the rhizome (Yob *et al.*, 2011). Nowadays, drug resistance of microorganisms was a major challenge for human. Therefore, the finding of novel bioactive compounds is increasingly desirable (Voravuthikunchai *et al.*, 2004). Even though some

plants are reported for their biology activities, but many of them still remain to be investigated that may be used as an effective drug reservoir for further development. However, genetics and environment of the plants may be importance factors in their biology activities.

Therefore, our major goals were to determine genetic variation of *Z. zerumbet* using RAPD technique and nuclear ribosomal DNA (ITS) and chloroplast DNA (trnL intron and trnL-F spacer) analysis, and to investigate for antioxidant and antibacterial activities. This molecular information may be useful for crop improvement to increase the efficiency of biological abilities.

Materials and Methods

Chemicals

Several chemicals were carried out for this research such as Folin-Ciocalteu's phenol reagent, gallic acid monohydrate and absolute ethanol (ACS grade) from Sigma-Aldrich (Steinheim, Germany), Ferric sulphate, potassium persulfate and D-glucose from Ajax Finechem (New South Wales, Australia). Ferrozine(3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine),

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2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) and EDTA from Fluka (Buchs, Switzerland). Glycerol and tryptone powder from Bio basic Inc, (Biotech grade), PureLink® Genomic DNA Kits from Invitrogen (USA), GoTaq® Green Master Mix from Promega, USA and GenUP™ PCR Cleanup Kit from Biotechrabbit, Germany.

Plant extraction

Fifteen native plants of *Zingiber zerumbet* were collected from the hilly region of Mahidol university, Kanchanaburi campus. The leaf parts of *Z. zerumbet* were washed and incubated at 55 °C until dried. Then, they were finely grinded by a homogenizer and extracted with 95% (w/v) ethanol. After that, one gram of the dried leaves was added to 10 ml of the solvent and stirred up at 37°C for 14 h, and the plant extracts were sieved using cheesecloth. All supernatants of each extract were concentrated at 45 °C under vacuum by rotary evaporator (BUCHI Rotavapor R-205), and adjusted volume to 1 g/ml with 95% ethanol, and then stored at -20°C until used for further tests.

DNA extractions and RAPD technique

DNA from each fresh leaf was isolated following the protocol of PureLink® Genomic DNA Kits (Invitrogen, USA). Then, each DNA sample was used as a template for PCR amplification by using an arbitrary primer (Ashraf *et al.*, 2014) based on the RAPD technique. A total 20 µl volume of PCR amplification consisted of 20 ng DNA (1 µl), 10 µl of GoTaq® Green Master Mix from Promega, USA (2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM of each dNTP and 3 mM MgCl₂), 5 µM primer for 4 µl and 5 µl of sterile water. PCR condition was performed by pre-denaturation at 94°C for 3 min, followed by 35 cycles of 94°C denaturation for 30 sec, annealing at 46°C for 45 sec and extension at 72°C for 60 sec. The final extension was at 72°C for 5 min. The PCR products were checked by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining under UV light.

Gene amplification and DNA sequencing

Genomic DNA was amplified with primer pairs (Taberlet *et al.*, 1991; Moller and Cronk, 1997; Rangsiruji, 1999) specific for TrnL-F spacer, TrnL intron and ITS genes according to the PCR condition of Ngamriabsakul (2000) with a few adjustment. Briefly, PCR was performed in a total volume of 50 µl containing 40 ng DNA (2.5 µl), 25 µl of GoTaq® Green Master Mix (Promega, USA), 1 µM of each

primer (6.25 µl) and 10 µl of sterile water. The initial PCR denaturation step was carried out at 95°C for 5 min followed by 35 cycles of 95°C for 30 min denaturation, 45°C for 45 sec annealing and 72°C for 60 sec extension. The final extension was made at 72°C for 7 min. The PCR products were checked similar to describe above, and purified with GenUP™ PCR Cleanup Kit (Biotechrabbit, Germany). The purified PCR products were sequenced by ABI Automatic Sequencer 3730XL under BigDye terminator cycling, Macrogen Inc. (Seoul, Korea). The sequence similarity was searched in the nucleotide-nucleotide BLAST program implemented in website: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch.

Total phenolic contents

The total phenolic contents in the plant extracts was measured with the Folin-Ciocalteu reagent following the protocol of Deetae *et al.* (2012). Gallic acid was used as standard. Folin-Ciocalteu's reagent (10 ml) and 7.5% Na₂CO₃ (0.8 ml) were mixed and incubated at 30°C for 1.5 h and measured the absorbance at 765 nm under visible spectrophotometer (Thermo Spectronic model 4001/4, USA)

ABTS radical scavenging activity

The ABTS radical scavenging assay was measured by following the protocol of Deetae *et al.* (2012). The ABTS radical cation was obtained from the reaction of 10 ml ABTS solution (7 mM) with 179 µl potassium persulfate (140 mM) in the dark for overnight. Before the assay, the ABTS radical cation was diluted with distilled water for an initial absorbance of 0.700 ± 0.050 at 734 nm. The ABTS radical scavenging assay was determined by mixing the prepared ABTS solution (3.9 ml) with the crude extract (20 µl) in quadruplicate. After the mixing solution was incubated in the dark at room temperature for 6 min, the absorbance was assessed at 734 nm by spectrophotometer (Thermo Spectronic model 4001/4, USA). The percentage antioxidant capacity was calculated using the formula following the previous report of Deetae *et al.* (2012) and EC₅₀ (50% effective concentration) values were calculated according the method of Thummajitasakul *et al.* (2014).

The ferrous ion-chelating (FIC) ability

Each concentrations of the crude extract (1 ml) were reacted with 1 ml of 0.1 mM FeSO₄ and 1 ml of ferrozine (0.25 mM), and incubated at room temperature for 10 min. After that, the absorbance was assessed at 562 nm. The test was accomplished

in quadruplicate. The percentage of metal chelating ability was estimated by the formula demonstrated by Deetae *et al.* (2012). The EC_{50} value was obtained using the above method.

Antibacterial activities

The 95% alcohol extracts from K373, K374 and K385 leaf samples were tested for antibacterial activities against five pathogens (*Escherichia coli* (TISTR780), *Staphylococcus aureus* (TISTR1466), *Staphylococcus epidermidis* (TISTR518), *Bacillus cereus* (TISTR687) and *Bacillus subtilis* (TISTR008) using the agar disk diffusion technique (Thummajitsakul *et al.*, 2012). The seven concentration (1,000, 500, 250, 125, 60.25, 30.125 and 15.06 mg/ml) of *Z. zerumbet* extracts were tested for antibacterial activities. Ampicillin (10 mg/ml) was used as positive control, and 95% alcohol was used as negative control. The dilution of each single colony of individual bacteria strain was spread on PCA plate. The sterilized paper disc (diameter 0.6 mm) was put on the PCA plate. Six μ l of each extract concentration was pipetted on the paper disc and incubated on 37 °C for 18 hours. The inhibition zone were obtained from the total diameter subtracted the diameter of paper disc.

Data analysis

Reproducible DNA bands on RAPD gel were counted as present (1) or absent (0), and recorded in a binary data. Consequently, the binary data was analyzed in MEGA 6.0 program (Tamura *et al.*, 2013).

Total phenolic contents, antioxidant and antibacterial properties of 95% ethanol extracts were revealed in mean and standard deviation. Analysis of variance (ANOVA) method was used to determine the difference among the ethanolic extracts at significant level of P-value < 0.05 (Soper, 2013). In addition, Cluster analysis (CA) was done by Multibase 2015, an Excel Add-In program.

Results and Discussion

Random amplified polymorphic DNA (RAPD) is one of popular used molecular markers to study genetic diversities in several organisms. This technique is applied from PCR reaction, and used rapidly, cheaply and easily, and no prior data of DNA sequences (Williams *et al.*, 1990). In our study, fifteen plant materials of *Z. zerumbet* were collected locally from the area of Mahidol University, Kanchanaburi campus, and an intraspecific genetic variation was determined by a RAPD technique using 12 arbitrary

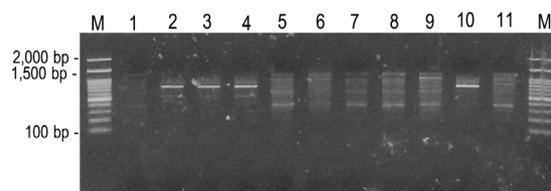


Figure 1. An example of RAPD bands for *Z. zerumbet* from OPA04 primer. A 100 bp was used as DNA marker.

Table 1. RAPD primers and genetic variation of *Z. zerumbet*

RAPD Primer	Size range of PCR products amplified (bp)	No. of reproducible bands	No. of haplotypes	Mean He	% polymorphic bands
OPA01	200-800	6	4	0.3472±0.0811	83.3
OPA03	400-1,500	5	5	0.1837±0.0916	80.0
OPA04	300-1,500	6	4	0.3625±0.0882	83.3
OPA09	300-1,500	4	4	0.2456±0.1418	50.0
OPA010	200-800	6	3	0.2282±0.0816	66.7
OPA011	500-1,000	3	3	0.1705±0.1125	66.7
OPA015	200-1,000	5	1	0	0

primers. The result showed that only 7 primers could provide RAPD bands (Figure 1). Among these primers, the OPA04 primer provided the most genetic variation within *Z. zerumbet* population (Mean He±SD: 0.3625±0.0882 and % polymorphic bands:83.3), followed by the results of OPA01, OPA03, OPA09, OPA010, OPA011 and OPA015, respectively (Table 1). The result provided a total of 35 bands and 11 haplotypes, and the intraspecific genetic variation was performed by the values of mean He±SD and % polymorphic bands (0.2297±0.0365 and 62.86%, respectively) representing moderate genetic diversity within the species. Besides, genetic distance between each individual ranged from 0.00 to 1.255.

The RAPD technique has been used to detect genetic polymorphisms within *Zingiber officinale* Rosc. Twenty arbitrary primers were used and among these primer, 13 primers provided 275 amplification products and % polymorphism in range from 88.23% to 100% (Ashraf *et al.*, 2014). Our results were concordant with earlier, RAPD markers could be used to detect the genetic diversity in ginger population (Mohd *et al.*, 2004; Palai *et al.*, 2007; Islam *et al.*, 2007), and helped to identify similar species of ginger *Lycium barbarum* (Zhang *et al.*, 2001).

To confirm RAPD results, the partial sequences of ITS, trnL intron and trnL-F spacer genes of *Z. zerumbet* were amplified by a PCR method and

Table 2. DNA sequences of nuclear ribosomal DNA (ITS) and chloroplast DNA (trnL intron and trnL-F spacer)

Parameter	ITS	TrnL intron	TrnL-F spacer	Total
Length range (total) (bp)	307*	300*	300*	907
G+C content range (%)	48.53-53.09	31.67-34.67	33-35.67	38.26-40.90
G+C content mean (%)	51.66	33.49	33.47	39.63
Transitions (unambiguous)	136.00	135.00	180.00	451.00
Transversions (unambiguous)	58.00	51.00	38.00	147.00
Transitions/transversions (ts/tv)	113.00	114.00	82.00	309.00
Number of variable sites (% in parentheses)	288 (93.81)	293(97.67)	275(91.67)	856(94.34)
Genetic distances	0.00-30.28	0.00-13.93	0.00-8.21	0.00-2.34

*307 and 300 bp is the length of ITS and TrnL genes set used in the sequence analyses, respectively

analyzed using BioEdit 7.2.5 and MEGA6.0 program. The results revealed 856 variable sites (or 94.34%), and 451 nucleotide transitions and 147 nucleotide transversions (Table 2). Then, the moderate level of genetic diversity was detected with the value of 1.032 corresponding with the RAPD results, and genetic distances were the range of 0.00 to 2.34.

Moreover, a neighbor-joining tree was generated from the binary data of RAPD bands that were amplified by all tested primers. All individuals of *Z. zerumbet* were divided into two main groups (group I and group II) shown in Figure 2A. This result corresponded to a neighbor-joining tree analyzed from the ITS, trnL intron and trnL-F spacer sequences. It also divided the tested samples into two main groups (group I and group II) shown in Figure 2B. It revealed that the variation of ITS, trnL intron and trnL-F spacer sequences were existence in *Z. zerumbet*. The result supported that the ITS, trnL intron and trnL-F spacer region could be used as a useful nuclear and chloroplast DNA marker in phylogenetic analysis.

Many evidences showed that ITS, trnL intron and trnL-F spacer sequences have been used to detect intraspecific variation in other Zingibereae, for example, *Gentiana L.* from the earlier finding of Gielly *et al.* (1996), and the ITS sequence has been used as popular molecule for phylogenetic analysis (Senchina *et al.*, 2003), though the sequences provided low genetic variation (Harris *et al.*, 2000). In our study, the ITS, trnL intron and trnL-F spacer analyses provided accurate trees and were used to support RAPD analyses. The RAPD analysis and the sequence data showed moderate genetic variation. The ITS, trnL intron and trnL-F spacer sequences provided more reliable data at fine levels in the phylogenetic tree.

The *Z. zerumbet* plants classified by genetic background were further investigated for the total phenolic contents, antioxidant and antibacterial

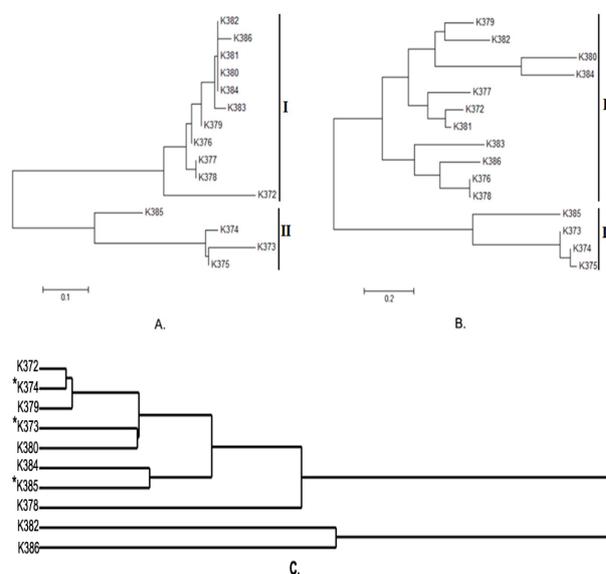


Figure 2. Phylogenetic tree based on neighbor-joining method. A was obtained from RAPD amplified products by 7 arbitrary primers and B was obtained from the ITS, trnL intron and trnL-F spacer sequences of *Z. zerumbet*. C was cluster analysis of total phenolic contents in selected tested plants using nearest neighbor method. Symbols* mean samples which are selected further to determine antioxidant activities and antibacterial activities.

activities. Cluster analyze were done through Multibase program (Figure 2C) using total phenolic contents in 95% alcohol extracts of 10 samples that were selected based on their genetic trees. It revealed two clusters, cluster I included samples K372, K373, K374, K378, K379, K380, K384 and K385 (the total phenolic contents were 4.94 ± 0.16 , 5.89 ± 0.16 , 4.90 ± 0.22 , 2.04 ± 0.10 , 4.62 ± 0.24 , 6.77 ± 0.50 , 8.45 ± 0.27 and 9.49 ± 0.08 mg gallic acid/g extract, respectively), and cluster II included samples K382 and K386, shown by high levels of total phenolic contents (15.24 ± 0.17 and 18.02 ± 1.11 mg gallic acid/g extract, respectively).

Then, K373, K374 and K385 leave samples were further chosen to determine antioxidant and antibacterial activities. The antioxidant activities

Table 3. Antioxidant activities and antibacterial activities of 95% ethanol extracts against five pathogens

Plant extracts	FIC EC ₅₀ (mg/ml)	ABTS EC ₅₀ (mg/ml)	Total Phenolic contents (mg gallic/g extract)	Inhibition zone (mm)* (1g/ml)				
				Bacterial strains				
				<i>E.coli</i>	<i>B.subtilis</i>	<i>B.cereus</i>	<i>S.aureus</i>	<i>S.epidermidis</i>
K373	4.93±0.14	12.74±0.04	5.89±0.16	0.86±0.01	3.82±0.37	2.39±0.04	4.70±0.14	0
K374	2.00±0.02	12.13±0.18	4.90±0.22	0.14±0.01	2.08±0.08	3.41±0.13	3.73±0.07	0
K385	3.14±0.03	11.92±0.13	9.49±0.08	1.06±0.01	1.29±0.10	2.80±0.25	2.75±0.31	0

of K373, K374 and K385 *Z. zerumbet* were determined using FIC and ABTS assays. The abilities of 95% ethanol extracts on chelating ferrous ion were demonstrated by the EC₅₀ values in the following order: 4.93±0.14, 2.00±0.02 and 3.14±0.03 mg/ml, respectively. The antioxidant activities determined with the ABTS method showed the EC₅₀ values in this order: 12.74±0.04, 12.13±0.18 and 11.92±0.13 mg/ml, respectively. Antibacterial activities of the ethanolic extracts from tested samples were tested against pathogens: *E. coli*, *B. subtilis*, *B. cereus*, *S. aureus* and *S. epidermidis*. It found the extracts were effective against both gram negative and positive bacteria pathogens, except for *S. epidermidis*. The MIC values of the extracts against each pathogen were 250, 250, 250 and 3.90 mg/ml, respectively (Table 3).

The results indicated that the samples from nearby sources were effect to biological activities of 95% ethanol extracts. Significant differences in biological activities among tested samples of *Z. zerumbet* in the area were found. The antioxidant activities of the 95% ethanol extracts were determined by FIC and ABTS assay. The leave extract of tested samples from nearby sources showed significantly different abilities in chelating ferrous ion and antioxidant activities (P-value<0.05). Moreover, the ethanolic extracts of tested samples were significantly different effective against both gram negative and positive bacteria (P-value<0.05): *E. coli*, *B. subtilis*, *B. cereus* and *S. aureus*.

Conclusions

The findings from this study indicated the existence of intraspecific genetic variation and the potential in natural antioxidant and antibacterial of *Z. zerumbet* for improving human health. These results may also be used to improve and manage conservative program for native ginger in selected regions, and to development increasing the efficiency

of biological abilities.

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